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Role of Cotranslational Disulfide Bond Formation in the Folding of the

LORI W. MCGINNES and TRUDY G. MORRISON[1]

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

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The role of cotranslational disulfide bond formation in the folding pathway of the hemagglutinin-neuraminidase (HN) glycoprotein of Newcastle disease virus was explored. Electrophoresis of pulse-labeled HN protein in the presence or absence of reducing agent showed that, characteristic of many glycoproteins, the nascent HN protein contains intramolecular disulfide bonds. As reported by Braakman *et al.* (*EMBO J.* 11, 1717–1722, 1992), incubation of cells in dithiothreitol (DTT) blocked the formation of these bonds. Removal of DTT after a pulse-label allowed for the subsequent formation of intramolecular disulfide bonds and folding of the molecule as assayed by the appearance of conformationally sensitive antigenic sites and by the formation of disulfide-linked dimers. However, the $t_{1/2}$ for the formation of a conformationally sensitive antigenic site after synthesis in the presence of DTT was over twice that of the control. Furthermore, the order of appearance of the antigenic sites was different from the control, suggesting that inhibition of cotranslational disulfide bond formation altered the folding pathway of the protein. Similar results were obtained in a cell-free system containing membranes. The HN protein forced to form intramolecular disulfide bonds posttranslationally had no detectable neuraminidase or cell attachment activity, suggesting that the protein had an abnormal conformation.

An essential element in the assembly of paramyxoviruses such as Newcastle disease virus (NDV) is the formation of spikes or peplomers. These structures, composed of homooligomers of the hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins, begin their assembly in the rough endoplasmic reticulum with folding of the monomer (Doms *et al.,* 1993; McGinnes *et al.,* 1985). The HN protein contains intramolecular disulfide bonds which form during folding (Iwata *et al.,* 1994; McGinnes and Morrison, 1994c). As suggested by early studies of the nonviral glycoproteins immunoglobulin and albumin, it is likely that some disulfide bonds form cotranslationally on the nascent polypeptide chain while others form posttranslationally (Bergman and Kuehl, 1979; Peters and Davidson, 1982). However, very little is understood about the role of these bonds in their folding. Recent reports from several laboratories have described a method to explore the importance of formation of disulfide bonds during translation (Braakman *et al.,* 1992; Lodish and Kong, 1993; Tatu *et al.,* 1993). These studies showed that incubation of cells in dithiothreitol (DTT) results in the synthesis of an unfolded protein lacking disulfide bonds. Upon removal of DTT, disulfide bonds form and the molecule folds such that oligomerization and intracellular transit can occur. Using this protocol, the role of cotranslational disulfide bond formation in the maturation of the HN protein of NDV was explored.

[1] To whom correspondence and reprint requests should be addressed. E-mail: Trudy.Morrison@BANYAN.UMMED.EDU.

The mature HN protein of the AV strain of NDV, a tetramer composed of two disulfide-linked dimers (Morrison and Portner, 1991), has 14 cysteine residues (McGinnes *et al.,* 1987). The most amino terminal cysteine is located in the cytoplasmic domain of this type 2 glycoprotein while the next most amino terminal cysteine, located just past the transmembrane domain, is responsible for the intermolecular disulfide bond which forms the covalently linked dimer (McGinnes and Morrison, 1994b). The 12 most carboxy terminal cysteine residues are likely involved in intramolecular disulfide bond formation. Mutation of any one of these twelve residues results in failure to form a conformationally normal, biologically active protein (McGinnes and Morrison, 1994c).

Our previous studies have defined a maturation pathway for the HN oligomer using conformationally sensitive monoclonal antibodies. Monoclonal antibodies define six to seven conformationally sensitive antigenic sites (Iorio *et al.,* 1989a). We have previously shown that these sites appear sequentially with time after synthesis and define a folding sequence for the molecule (McGinnes and Morrison, 1994a). Furthermore, proteins mutated in individual cysteine residues are blocked at different stages in this pathway (McGinnes and Morrison, 1994c). Definition of this pathway has allowed us to determine if inhibition of cotranslational disulfide bond formation alters the folding sequence of this protein. Furthermore, we have determined if the HN protein forced to form disulfide bonds posttranslationally can acquire biological activity. We

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present evidence that forcing the HN protein to form all disulfide bonds posttranslationally resulted in an altered folding pathway and a protein with no detectable activity.

MATERIALS AND METHODS

Cells and viruses

Cells used were Cos-7 cells (obtained from the American Type Culture Collection) maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, glutamine, vitamins, penicillin/streptomycin, and 10% fetal calf serum.

NDV, strain Australia-Victoria (AV), was grown and purified as previously described (Morrison *et al.,* 1985).

Infection and radioactive labeling of cells

Confluent monolayers (2×10^6 cells) were infected at a multiplicity of 10 PFU per cell. After 5 hr of incubation at 37°, the medium was removed and the monolayers were washed three times with methionine-free minimal essential medium (supplemented with nonessential amino acids and 10% dialyzed fetal calf serum). Express [^{35}S]methionine (110 μC/ml, 11,175 Ci/mmol; New England Nuclear Corp.) in methionine-free media was added to the cells and the monolayers were incubated at 37° for 5 min. The labeling medium was removed and chase medium was added. Chase medium was DMEM supplemented with 100 μg/ml cycloheximide, 2 m*M* methionine, nonessential amino acids, vitamins, glutamine, penicillin/streptomycin, and 10% fetal calf serum.

Cells treated with dithiothreitol (DTT) during the pulse-label were first washed with methionine-free DMEM and incubated in methionine-free DMEM containing 5 m*M* DTT for 5 min and then methionine-free DMEM containing DTT and [^{35}S]methionine for 5 min. Cells were washed and incubated in complete media containing cycloheximide at 37 or 15°. For incubation with DTT just after the pulse-label, cells were radioactively labeled as above except with the omission of DTT. After the pulse label, cells were incubated for 5 min at 37° in complete media containing DTT and cycloheximide, washed, and then incubated at 37 or 15° in compete media without DTT but containing cycloheximide. For cells chased in the presence of DTT, pulse-labels and nonradioactive chases were accomplished as above except that DTT (5 m*M*) was present throughout.

At the end of the chase, cells were washed in PBS containing 5 m*M* *N*-ethylmaleimide (NEM) and then incubated in PBS containing 5 m*M* NEM for 1 min. Cells (2×10^6) were lysed in 0.5 ml of RSB (10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10 m*M* Tris–hydrochloride (pH 7.4)) containing 2 m*M* iodoacetamide, 2.5 m*M* NEM, 1% Triton-X100, and 0.5% sodium deoxycholate. Nuclei were removed by centrifugation.

Antibodies

Monoclonal antibodies were the generous gift of Dr. Ron Iorio and have been previously characterized (Iorio *et al.,* 1989a, 1989b, 1992, 1991). Antibody specific to sites, 2, 3, 4, and 14 were anti-2b, anti-3a, anti-4a, and anti-14c, respectively. Anti-NDV is a polyclonal rabbit antibody raised against UV-inactivated virions and contains antibody specific for only the mature form of the HN protein (McGinnes and Morrison, 1994c). Anti-AC and anti-AS are polyclonal rabbit antibodies raised against peptides derived from the HN protein as previously described (McGinnes and Morrison, 1994c) and react only with nascent or unfolded HN protein (McGinnes and Morrison, 1994c).

Immunoprecipitations

Cell extracts were incubated with excess antisera for 1 hr at room temperature. Immune complexes present were precipitated with Staphlococcus aureus protein A-positive cells (Boehringer/Mannheim Corp.) as previously described (McGinnes and Morrison, 1994a). Immune complexes were solubilized by boiling in 4% sodium dodecyl sulfate and solubilized proteins were resolved in 10% polyacrylamide slab gels as previously described (Morrison and McGinnes, 1989; Morrison *et al.,* 1990).

Immunoprecipitation of cell surface molecules

Cells infected for 5 hr were radioactively labeled with [^{35}S]methionine for 5 min and chased for various lengths of time in nonradioactive medium. Anti-NDV antibody or monoclonal antibody was incubated with intact cells and excess unbound antibody removed as previously described (McGinnes and Morrison, 1994c; Morrison *et al.,* 1990). After cell lysis, proteins present in recovered immune complexes were electrophoresed on 10% polyacrylamide gels. Mixing experiments accomplished as previously described (Morrison *et al.,* 1990) guaranteed that all labeled HN protein precipitated was cell surface HN protein.

Cell-free protein synthesis

Capped HN mRNA was generated using SP6 polymerase (Promega) by protocols outlined by Promega Corp. Cell-free protein synthesis was accomplished using rabbit reticulocyte extracts (Promega) containing dog pancreas membranes (generous gift of Dr. Reid Gilmore). Each 25-μl reaction contained 17.5 μl lysate, 0.5 μl RNasin (Promega), 2 μl [^{35}S]methionine (100 μCi/ml, 1400 Ci/m*M,* New England Nuclear), 1 μl mRNA, 100 m*M* potassium acetate, 1 μl membranes, and glutathione (Sigma) (0–3 m*M*). Incubation at 30° was terminated by the addition of NEM (24 m*M*).

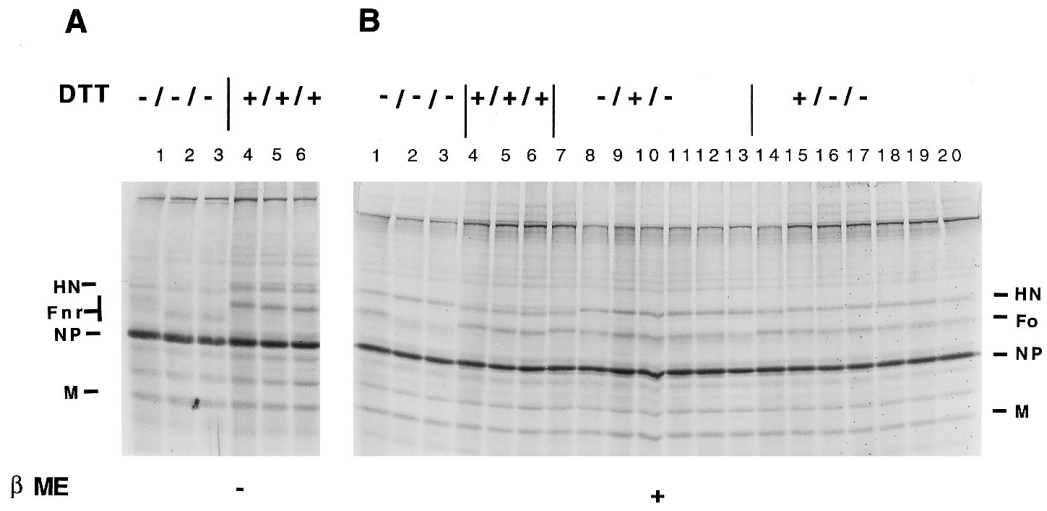

FIG. 1. Synthesis and stability of the HN protein in the presence of DTT. Cos cells infected with NDV for 5.5 hr were pulse-labeled with [^{35}S]methionine for 5 min and chased for various times at 37° in the absence (−/−/−) (lanes 1–3) or presence (+/+/+) (lanes 4–6) of DTT. Infected cells were also pulse-labeled in the presence of DTT but chased in the absence of DTT (+/−/−) (lanes 14–20) or pulse-labeled in the absence of DTT, incubated for 5 min in DTT, and then chased in the absence of DTT (−/+/−) (lanes 7–13). Nonradioactive chases were for 5 min (lanes 8 and 15), 10 min (lanes 9 and 16), 15 min (lanes 10 and 17), 20 min (lanes 11 and 18), 30 min (lanes 2, 5, 12, and 19), and 60 min (lanes 3, 6, 13, and 20). At various times during the protocol cells were lysed in 1% Triton X-100/0.5% DOC as described under Materials and Methods. Total cytoplasmic extracts from equivalent numbers of cells were electrophoresed in the absence (A) or presence (B) of β-mercaptoethanol (β me). Autoradiographs shown in A and B were derived from two different but identical gels electrophoresed at the same time in order to avoid effects of diffusion of β-mercaptoethanol on nonreduced samples. HN, hemagglutinin-neuraminidase protein; F_0, uncleaved fusion protein; Fnr, nonreduced fusion protein composed of F_0 in pulse labels or disulfide linked F_1 and F_2 as well as F_0 in chases; NP, nucleocapsid protein; M, membrane protein. In the presence of β me, F_1 comigrates with the NP protein and F_2 is not resolved on this gel.

Assay of neuraminidase and attachment activity

Neuraminidase activity at the surfaces of infected cells was assayed as detailed previously (Morrison and McGinnes, 1989) using fetuin as the substrate.

Attachment, assayed as avian red blood cell binding to infected cell surfaces, was measured as previously described (Morrison and McGinnes, 1989) by quantitating hemoglobin released from bound red blood cells.

RESULTS

Intramolecular disulfide bonds in nascent protein

Electrophoresis of proteins under nonreducing conditions has been used to characterize intramolecular disulfide bond formation in several viral glycoproteins since proteins containing intramolecular disulfide bonds migrate more rapidly than totally reduced proteins due to a more compact structure (Braakman *et al.,* 1992; Doms *et al.,* 1993; Mulvey and Brown, 1994). To determine if the HN protein forms intramolecular disulfide bonds on the nascent protein, infected cells were pulse-labeled for 5 min and electrophoresed in the absence (Fig. 1A, lane 1) or presence of reducing agent (Fig. 1B, lane 1). Clearly, the reduced form of the pulse-labeled protein migrated slower than the bulk of the protein electrophoresed under nonreducing conditions, arguing that the protein acquires intramolecular disulfide bonds during or just after synthesis.

Synthesis and stability of the HN protein in the presence of DTT

To determine if exogenously added DTT blocks the formation of these rapidly acquired intramolecular disulfide bonds, cells were pulse-labeled in the presence of DTT. Clearly, HN protein pulse labeled under these conditions migrated, under nonreducing conditions, similarly to the fully reduced protein (compare Fig. 1A, lane 4 to Fig. 1B, lane 1) showing that DTT blocked intramolecular disulfide bond formation in nascent protein.

To determine the effect of exogenously added DTT on the stability of viral glycoproteins, infected cells were pulse-labeled and then chased in the absence (−/−/−) or presence of DTT (+/+/+) (Fig. 1B, lanes 1–3 and lanes 4–6, respectively) or pulsed-labeled in the presence of DTT and then chased in the absence of DTT (+/−/−) (Fig. 1B, lanes 14–20). In a number of different experiments, DTT, when added during synthesis, had a variable effect on the synthesis of all viral proteins, including the HN protein, compared with control-infected cells, varying from no inhibition to fourfold inhibition. However, the HN protein made in the presence of DTT was stable during a chase whether or not DTT was removed (lanes 4–6 and 14–20). Similarly, if DTT was added briefly after the pulse-label and then removed (−/+/−, Fig. 1B, lanes 7–13), there was no effect on the stability of the protein. However, the size of the HN protein chased in the presence of DTT was slightly smaller

than HN protein chased without DTT (Fig. 1B, lanes 3 and 6). This difference is consistent with a block in intracellular transport and, therefore, addition of peripheral sugars in Golgi membranes in the presence of DTT. Removal of DTT in the chase resulted in a normal sized HN protein, suggesting that processing of the protein's oligosaccharides can proceed as has been reported with other glycoproteins (lanes 14–20) (Braakman *et al.,* 1992; Tatu *et al.,* 1993).

Formation of conformationally sensitive antigenic sites

To determine the effect of synthesis of the HN protein in the presence of DTT on subsequent folding, the formation of conformationally sensitive antigenic sites was explored. We have previously shown that representative antibodies against different sites on the HN protein are unreactive to pulse-labeled HN protein but are reactive to protein subjected to a pulse-chase labeling protocol (McGinnes and Morrison, 1994a) (illustrated in Figs. 2A and 2B, lanes 2, 3, 10, and 11). Cells pulse-labeled and then chased in the presence of DTT contained HN protein which remained unreactive to four representative monoclonal antibodies (Figs. 2A and 2B, lanes 4, 5, 12, and 13), demonstrating that DTT blocked the formation of antigenic sites. However, protein made in the presence of DTT but chased in the absence of DTT acquired all four antigenic sites (Figs. 2A and 2B, lanes 6, 7, 14, 15), suggesting that the protein can fold if disulfide bonds are forced to form posttranslationally. Similarly, if pulse-labeled cells were subjected to a short incubation in DTT posttranslationally, the nascent-labeled proteins acquired antigenic sites upon removal of the reducing agent (Figs. 2A and 2B, lanes 8, 9, 16, and 17).

Efficiency and kinetics of folding

To explore the efficiency of folding, cells pulse-labeled in the absence or presence of DTT were subjected to a very long chase (4 hr) and then precipitated with three representative monoclonal antibodies (Fig. 3, lanes 1, 4, 7, 10, 13, and 16). The supernatants of the precipitations were then immunoprecipitated with a mix of polyclonal antibodies specific for nascent protein. No additional protein was precipitated (lanes 3, 6, 9, 12, 15, 18), showing that no unfolded protein remained. Since electrophoresis of HN protein in total extracts (Fig. 1) showed little evidence of protein degradation, it is likely that protein folding was efficient. Furthermore, the amount of HN protein precipitated in the −/+/− cells was similar to that precipitated from control cells, and the amount precipitated from the +/−/− cells was also similar to the controls after correction for incorporation.

To explore the kinetics of folding of the HN protein made in the presence of DTT, the amount of material precipitable with a representative monoclonal antibody

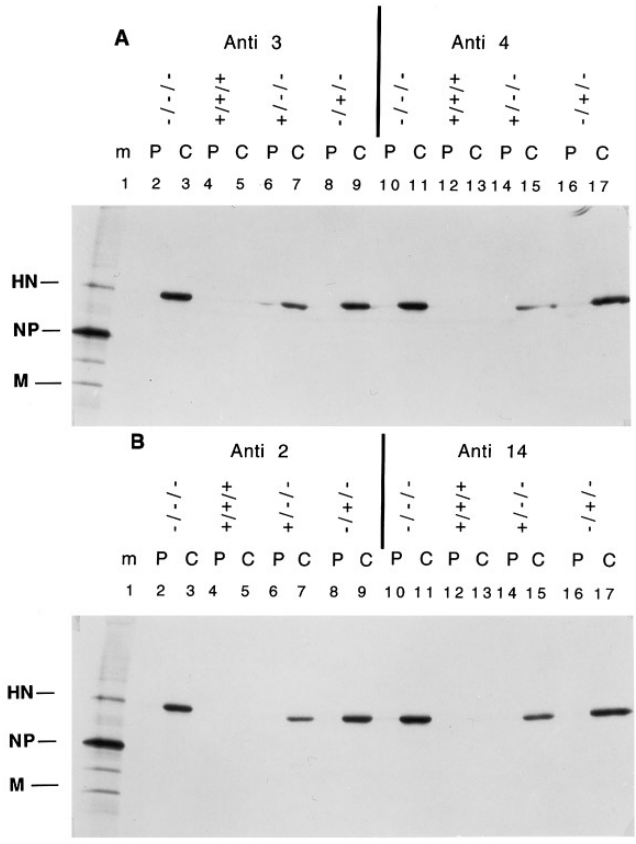

FIG. 2. Formation of conformationally sensitive antigenic sites in the presence and absence of DTT. Infected cells were pulse labeled for 5 min (P) or pulse-labeled for 5 min and chased for 2 hr at 37° in the absence (−/−/−) or presence (+/+/+) of DTT. Infected cells were also pulse-labeled in the presence of DTT but chased in the absence of DTT (+/−/−) or pulse-labeled in the absence of DTT, incubated for 5 min in DTT, and then chased in the absence of DTT (−/+/−). HN protein in each extract was immunoprecipitated with anti-3 (A, lanes 2–9), anti-4 (A, lanes 10–17), anti-2 (B, lanes 2–9), and anti-14 (B, lanes 10–17). Lane 1 contained total infected cytoplasmic extract as marker proteins (m). HN, hemagglutinin-neuraminidase protein; NP, nucleocapsid protein; M, membrane protein.

at various times after removal of DTT was compared to that present in control cells. In three separate experiments, the $t_{1/2}$ for the formation of antigenic site 4 in control cells at 37° was approximately 20 to 30 min (McGinnes and Morrison, 1994a). In contrast, the $t_{1/2}$ for the formation of site 4 in molecules made in the presence of DTT was approximately 60–70 min. Molecules made in the absence of DTT but subjected to a short incubation in DTT just after synthesis had a $t_{1/2}$ of approximately 50–60 min. Figure 4A shows a typical experiment demonstrating that reactivity of antibody to site 4 appeared much more slowly if the molecules were synthesized in the presence of DTT or if the protein was subjected to a short DTT incubation just after synthesis. Quantitation of the experiment shown in Fig. 4A is shown in Fig. 4B. Thus inhibition of cotranslational disulfide bond formation considerably slowed the subsequent folding of the protein after removal of DTT.

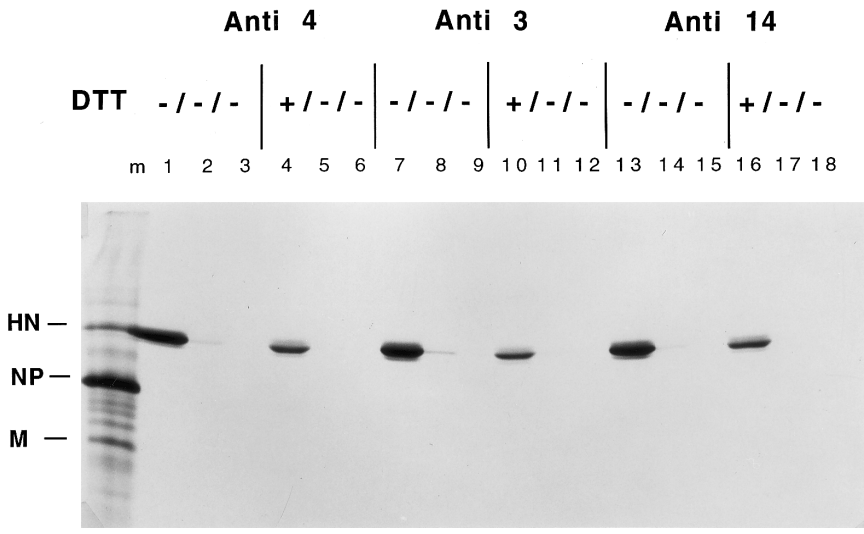

FIG. 3. Efficiency of folding of HN protein synthesized in the presence of DTT. Infected cells were pulse-labeled and chased at 37° in nonradioactive media for 4 hr in the absence of DTT (−/−/−) or pulse-labeled in the presence of DTT and chased for 4 hr in the absence of DTT (+/−/−). HN protein present in each extract was precipitated with anti-4 (lanes 1 and 4), anti-3 (lanes 7 and 10), and anti-14 (lanes 13 and 16). Supernatants containing nonprecipitated protein were again incubated with anti-4 (lanes 2 and 5), with anti-3 (lanes 8 and 11), and anti-14 (lanes 14 and 17). Supernatants containing nonprecipitated protein were incubated with a mix of anti-AC and anti-AS and the precipitated proteins are shown in lanes 3, 6, 9, 12, 15, and 18.

Oligomer formation

The HN protein forms a dimer due to the covalent linkage of cysteine residues near the transmembrane domain. This intermolecular disulfide bond forms posttranslationally after the appearance of antigenic site 4 as illustrated in Fig. 5A. Molecules synthesized in the presence of DTT (Fig. 5C) or molecules treated with DTT just after synthesis (Fig. 5B) also formed these dimers but again with delayed kinetics.

Order of appearance of antigenic sites

We have previously defined a folding pathway for the HN protein using the conformationally sensitive monoclonal antibodies described above. We have defined four steps in the folding of the HN protein by the order of appearance of the conformationally sensitive antigenic sites. In a pulse-chase labeling protocol, antigenic site 4 appeared first, followed by site 3 and site 1, then site 14, followed by site 2 (illustrated in Fig. 6A) (McGinnes and Morrison, 1994a). To determine if inhibition of cotranslational disulfide bond formation altered the order of appearance of antigenic sites, cells were pulse-labeled in the presence of DTT and then chased in the absence of DTT and the order of appearance of sites was compared with the untreated controls. Figure 6B shows that like the control, antigenic site 4 appeared first, followed by site 14. Surprisingly, and in contrast to the control, antigenic site 3 appeared after antigenic sites 14 and 2. Site 1, which appeared with site 3 in the control (McGinnes and Morrison, 1994a), also appeared after site 2 in cells treated with DTT (not shown). Thus, the order of appearance of antigenic sites was altered if cotranslational disulfide bond formation is inhibited. Similar results were obtained if chases were done at 37° (Fig. 6C).

If DTT is added briefly after the pulse label, the order of sites was also altered from the control (Fig. 6D). Again site 3 appeared after sites 2 and 14 as well as site 4.

Posttranslational folding in a cell-free system

The order of appearance of antigenic sites was also explored in a cell-free system. It has been previously shown that inclusion of glutathione (GSSG) in a cell-free system containing membranes will allow the formation of disulfide bonds and, therefore, folding of a protein (Scheele and Jacoby, 1982). Indeed, Fig. 7 shows that increasing amounts of glutathione up to 3 m*M* in a reticulocyte cell-free system containing dog pancreas membranes resulted in the synthesis of an HN protein increasingly reactive to conformationally sensitive antibodies (Figs. 7B and 7C) and decreasingly reactive to antibodies specific for unfolded protein (Fig. 7A). To determine the order of appearance of antigenic sites if GSSG is added posttranslationally, proteins were synthesized for 1 hr in the absence of GSSG to allow accumulation of protein without intramolecular disulfide bonds. Protein synthesis was halted with cycloheximide and glutathione was added to stimulate disulfide bond formation. Figure 8 shows the kinetics of appearance of antigenic sites after glutathione addition. As in intact cells released from DTT, site 4 appeared first, followed closely by antigenic sites 14 and 2. Antigenic site 3 appeared last. Thus the order of appearance of antigenic sites in a cell-free

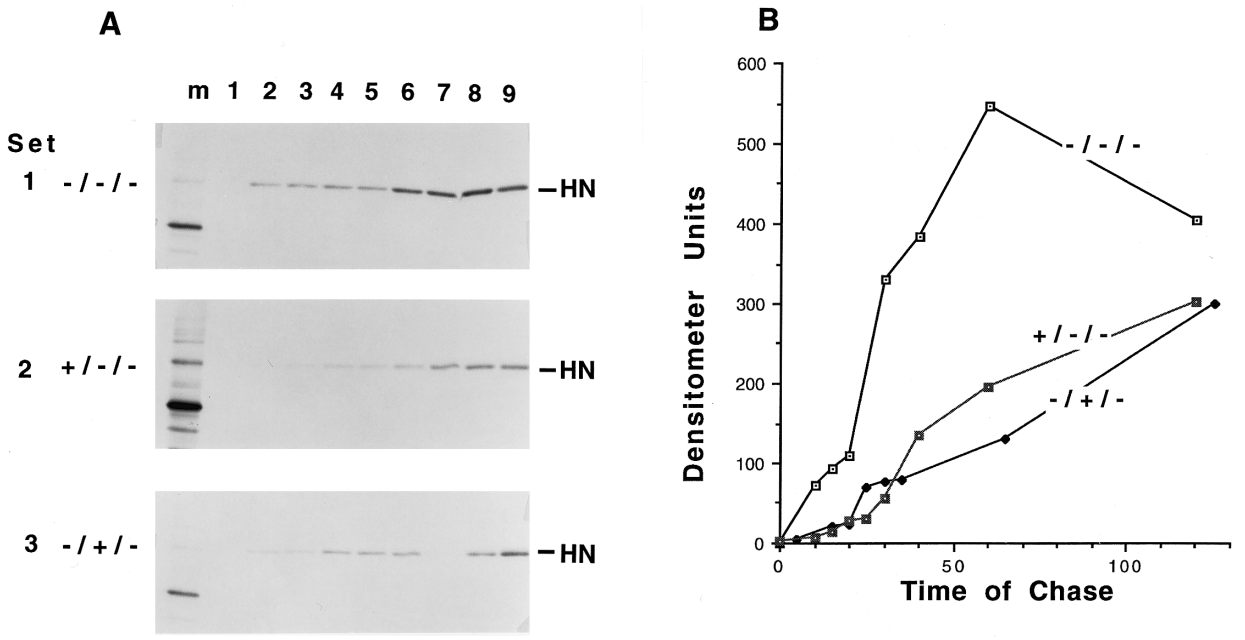

FIG. 4. The effect of DTT on the kinetics of folding. Infected cell monolayers were radioactively labeled in the absence or presence of DTT for 5 min and then subjected to various lengths of a nonradioactive chase at 37° in the absence of DTT (−/−/− and +/−/−). Another set of infected cell monolayers were pulse-labeled in the absence of DTT, incubated for 5 min in DTT, and then chased in the absence of DTT (−/+/−). HN protein in the resulting cell extracts was precipitated with anti-4 and the protein precipitated is shown in A. The autoradiograph in sets 1 and 3 were exposed for 6.5 hr while the autoradiograph in set 2 was exposed for 24 hr in order to compensate for a fourfold reduction in incorporation of label in the presence of DTT (determined by electrophoresis of total cell extracts). Lanes 1–9 show chases of 0, 10, 15, 20, 25, 30, 40, 60, and 120 min, respectively. The signal in each lane was quantitated using a Molecular Dynamics densitometer and the results are shown in B. Similar results were obtained by exposing the autoradiographs for the same times and normalizing the data to the levels of total protein synthesis.

system which allowed only posttranslational disulfide bond formation was similar to intact cells treated with DTT during synthesis.

Transport of HN to the cell surface

To determine if synthesis in DTT had any effect on subsequent intracellular transport of the HN protein, surface associated HN proteins were immunoprecipitated at various times of chase. Figure 9A, shows a typical result. After 2.5 hr of chase, the amount of HN detected at the surface of +/−/− cells was between 20 and 25% of the control amounts (Fig. 9A), a decrease that is accounted for by the decrease in incorporation of label in the presence of DTT. Furthermore, the appearance of HN at the surface was delayed consistent with slower folding of the protein after synthesis in the presence of DTT. Figure 9B, shows a quantitation of the results of three separate experiments. The values for the +/−/− conditions have been normalized to account for a fourfold reduction in incorporation. The $t_{1/2}$ for the surface incorporation in +/−/− cells is approximately 30 min longer than in −/−/− cells. Since the difference in the $t_{1/2}$ for the formation of antigenic site 4 is also approximately 30 min, the delay in appearance at the surface can be totally accounted for by a delay in folding.

Activities of HN protein

Since the folding of the HN protein after removal of DTT was slower than normal and since the order of appearance of antigenic sites was altered, it seemed possible that the HN protein was not folded entirely normally after removal of DTT. The ultimate test of a protein conformation is the biological activity of the protein. The HN protein has three activities, neuraminidase, attachment, and fusion promotion (Morrison and Portner, 1991; Sergel *et al.,* 1993). Neuraminidase can be assayed as an enzyme activity associated with the surfaces of intact cells using fetuin as a substrate. Attachment is measured by the binding of avian red blood cells to surfaces of cells expressing the HN protein. To measure neuramindase and attachment activity after synthesis of the HN protein in the presence of DTT, cells infected for 4 hr were treated using the protocols outlined in Fig. 10A. After 4 hr of infection, significant viral protein synthesis is just beginning and there are only small amounts of biologically active HN protein at the cell surfaces (Morrison and McGinnes, 1989) (Fig. 10B). Incubation of cells in DTT at this point in infection reduced the neuraminidase activity detected by only 25%, suggesting that mature HN is relatively insensitive to this concentration of reducing agent (Fig. 10B). No attachment activity was detected under any conditions at 4 hr postinfection (Morrison and McGinnes,

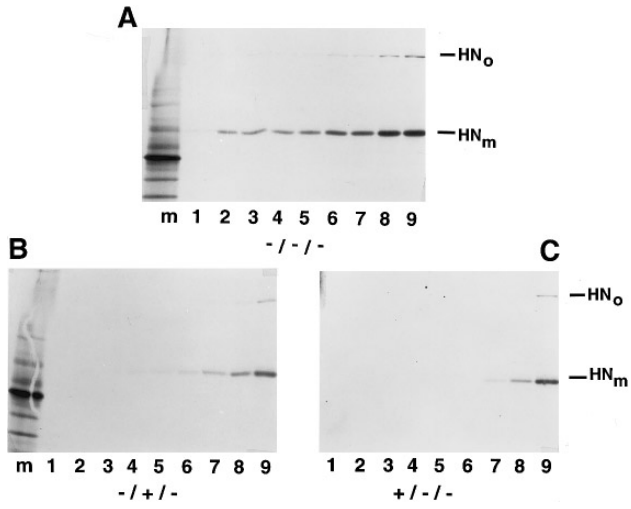

FIG. 5. Oligomer formation. Cell extracts were prepared as described in the Legend to Figure 4 except the nonradioactive chase was accomplished at 15°. Proteins precipitated from each extract were electrophoresed in the absence of reducing agent in order to resolve the HN dimer (HNo) from the HN monomer (HNm). Lanes 1–9 show extracts from cells chased 0, 10, 15, 20, 25, 30, 40, 60, 120 min, respectively. (A) no DTT; (B) DTT added after the 5 min pulse; (C) DTT present during the 5-min pulse. The autoradiograph shown in C was exposed for 4 days while those in A and B for 1 day.

1989). DTT was added to cells at 4 hr postinfection and synthesis of the viral proteins in the presence of DTT was allowed to proceed for 1.5 hr to allow unfolded HN protein to accumulate in the RER (protocol D). That the HN protein made under these conditions was stable is shown in Fig. 1, lanes 4–6. Controls done in parallel were: (1) no treatment of monolayers for this time period (protocol A), (2) monolayers treated with cycloheximide from 4 to 5.5 hr of infection, which completely inhibited protein synthesis as determined by polyacrylamide gel electrophoresis of cytoplasmic extracts (protocol B), and (3) monolayers treated with monensin from 4 to 5.5 hr of infection (protocol C). In the presence of monensin, the protein should accumulate inside cells but in a folded form (McGinnes and Morrison, 1994a; Morrison *et al.,* 1985). After 1.5 hr DTT and monensin were removed from the parallel cultures. All cells were further incubated in the presence of cycloheximide to allow transit of accumulated HN protein to the cell surface while blocking further synthesis of protein after removal of the DTT or monensin. The neuraminidase activity and cell attachment activity at the surfaces of each set of cells is shown in Figs. 10C and 10D, respectively. Clearly the activities found at the surfaces of cells treated with DTT were considerably lower than untreated cells or cells treated with monensin. Most significantly, the activities were even slightly lower than activities associated with cells treated with cycloheximide throughout the protocol. This result was observed in four separate experiments. Even accounting for a fourfold decrease in total protein synthesis in the presence of DTT seen in some experiments, there should be some activity of DTT-treated cells over cycloheximide-treated cells if the molecules had neuraminidase or attachment activity.

That prolonged treatment of cells in DTT did not cause significant irreversible aggregation of the HN protein was shown by precipitating HN protein from extracts derived from protocols A and D with anti-4 antibody. There was a four- to fivefold decrease in precipitable material after DTT treatment which can be accounted for by the decrease in total protein synthesis. Indeed, if the precipitated protein was biologically active, the level of activity that would be detected is shown in the last column of Figs. 10C and 10D (exp DTT).

The small increase in neuraminidase activity observed on cells treated with cycloheximide from 4 to 8 hr after infection (protocol B) was likely due to transport to the surface of HN made prior to cycloheximide addition. Similarly, the small increase in activity from 4 to 8 hr on cells treated with DTT can be accounted for by the transport to the surface of HN protein already folded before DTT treatment. Thus, neuraminidase and cell attachment activities of the HN protein synthesized in the presence of DTT could not be detected.

The number of infectious virus released from cells during this protocol was also determined (Table 1). In two separate experiments, the plaque forming units (PFU) released from cells treated with DTT was slightly less than that released from cycloheximide-treated cells. Lack of PFU released under these conditions could be due to absence of attachment activity of the HN protein.

DISCUSSION

The formation of mature, oligomeric glycoproteins are the result of a complex pathway which begins with synthesis and folding in the rough endoplasmic reticulum (Doms *et al.,* 1993). In our studies of the maturation of

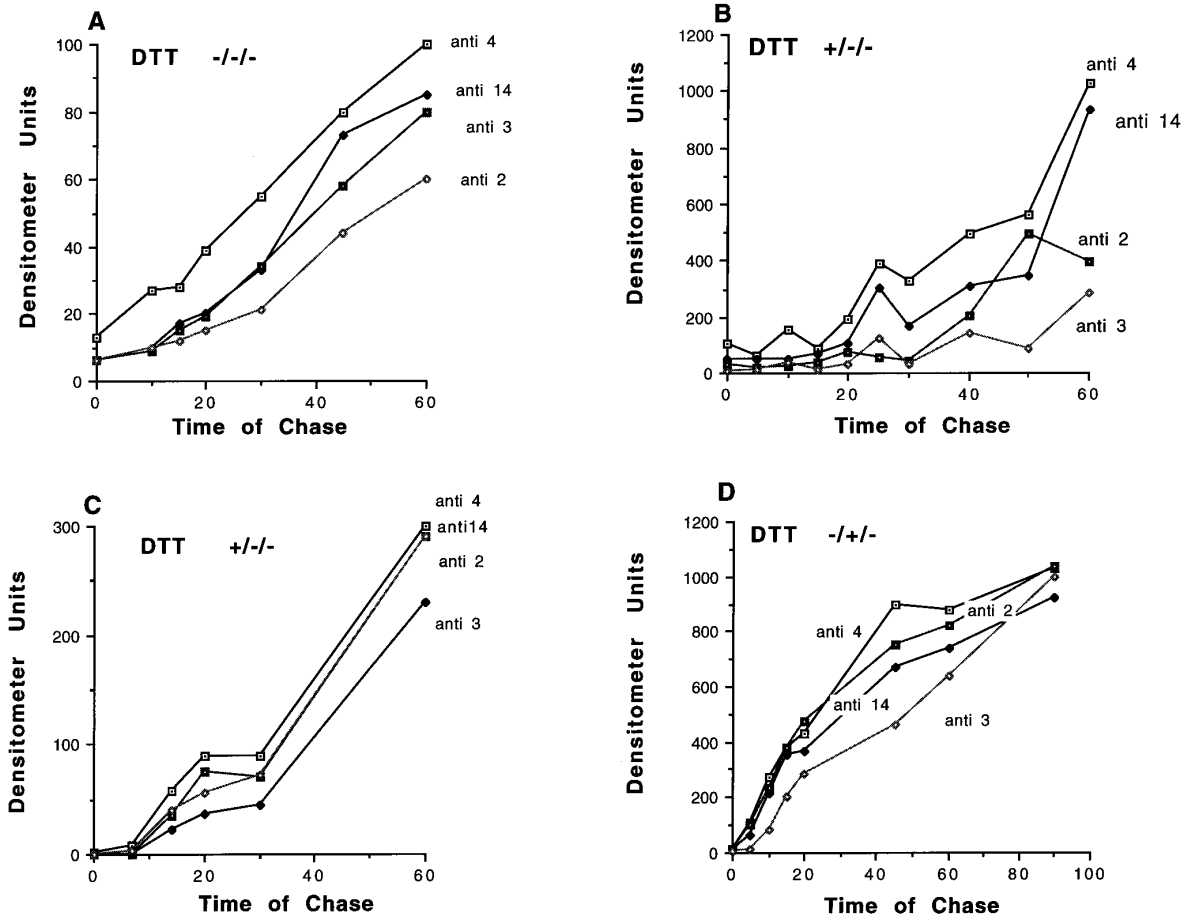

FIG. 6. The effect of DTT on the order of appearance of antigenic sites. Infected cell monolayers were radioactively labeled in the absence or presence of DTT for 5 min and then subjected to various lengths of a nonradioactive chase in the absence of DTT as described in the legend to Fig. 4. HN protein present in total cytoplasmic extracts was precipitated with anti-4, anti-3, anti-2, and anti-14 as described in the legend to Fig. 2. The amount of HN protein precipitated at each time point was quantitated using a Molecular Dynamics densitometer as described in the legend to Fig. 4. (A) The order of appearance of sites during the chase at 15° in cells not treated with DTT as previously reported (McGinnes and Morrison, 1994a). (B) The order of appearance of sites in cells pulse-labeled in DTT and chased in the absence of DTT at 15°. (C) A similar experiment as in B except chases were at 37°. (D) The results of chases of cells treated for 5 min DTT after the pulse label.

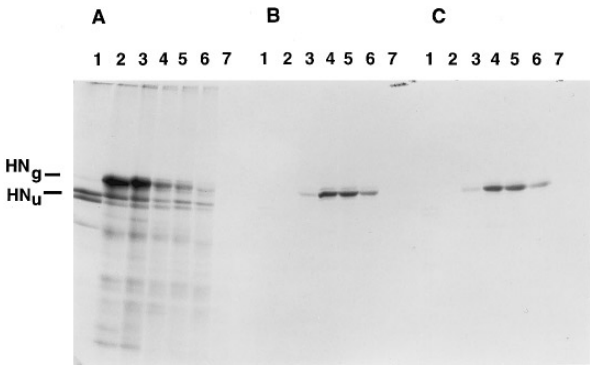

FIG. 7. GSSG promotes the formation of antigenic sites in a cell-free system. HN mRNA was translated in a rabbit reticulocyte cell-free system in the absence (lane 1) or presence of dog pancreas membranes (lanes 2–7) and various concentrations of GSSG (lane 2, 0 m*M;* lane 3, 0.5 m*M;* lane 4, 1 m*M;* lane 5, 2 m*M;* lane 6, 3 m*M;* lane 7, 4 m*M*). Proteins made in each reaction were precipitated with a mix of anti-AC and anti-AS (A), with anti-NDV (B), and anti-4 (C).

the HN protein of NDV, we have shown that mature, conformationally sensitive antigenic sites form posttranslationally in this organelle (McGinnes and Morrison, 1994a). By determining the kinetics of formation of each of these sites, we have defined at least four steps in the folding of the molecule (McGinnes and Morrison, 1994a). Formation of intramolecular disulfide bonds during this folding pathway is likely important since mutation of individual cysteine residues blocks maturation at different steps in the pathway (McGinnes and Morrison, 1994c). At least some of the intramolecular disulfide bonds likely form either cotranslationally or just after the completion of synthesis of the molecule since the migration of pulse-labeled protein on gels in the absence of reducing agent suggests a more compact structure than the fully reduced protein.

There have been two views on the order of formation of disulfide bonds. Studies of small, secreted proteins suggested that the folding process involves intermedi-

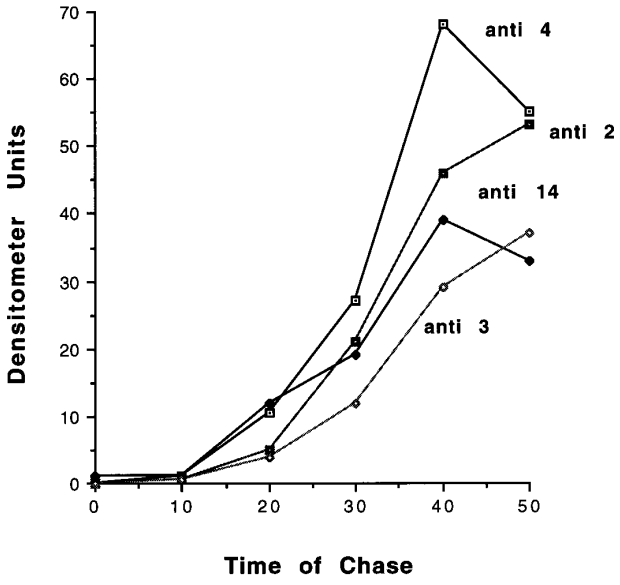

FIG. 8. Order of appearance of antigenic sites upon posttranslational addition of GSSG. HN mRNA was incubated in a rabbit reticulocyte lysate containing dog pancreas membranes for 1 hr. Cycloheximide and 2 m*M* GSSG was then added and incubation was continued and aliquots of the reaction removed with time after the addition of GSSG and cycloheximide. The HN protein in separate aliquots of the reaction was precipitated with anti-4, anti-3, anti-14, and anti-2 and the precipitated protein resolved on polyacrylamide gels. The amount of radioactively labeled HN protein precipitated in each aliquot was quantitated with a densitometer as described in the legend to Fig. 4.

ates containing nonnative disulfide bonds which are broken as the protein matures (Creighton, 1988; Kim and Baldwin, 1990). In this case, formation of disulfide bonds cotranslationally may not be an absolute requirement for subsequent folding. More recent work of Weissman and Kim (1991), however, has suggested that the major folding intermediates contain only native disulfide bonds. In this scenario, it seems possible that, in some cases, formation of disulfide bonds cotranslationally may be necessary for subsequent folding. To determine if cotranslational disulfide bond formation influences the subsequent folding pathway of the HN protein, use was made of the observation of Brakmaan *et al.* (1992) that incubation of intact cells in low concentrations of DTT blocks formation of intramolecular disulfide bonds during synthesis and also disrupts these bonds in newly made protein. Indeed, we found that the HN protein made in the presence of DTT and chased in the presence of DTT failed to form all mature antigenic sites, suggesting that the reducing agent prevented the folding of the HN protein and that disulfide bond formation is critical to HN maturation as previously suggested by our analysis of mutations in cysteine residues. It has been shown that DTT treatment has no detectable affect on the transport machinery of the cell (Lodish and Kong, 1993; Opstelten *et al.,* 1993).

After synthesis in the presence of DTT, several proteins, including the influenza HA glycoprotein and the coronavirus S glycoprotein, form conformationally sensitive antigenic sites and are transported to the cell surface after removal of DTT (Braakman *et al.,* 1992; Opstelten *et al.,* 1993). Similarly, HN protein synthesized in the presence of DTT formed conformationally sensitive antigenic sites after the removal of DTT. In addition, the molecule formed disulfide linked dimers. Furthermore, the efficiency of formation of antigenic sites and oligomers appeared similar to normal since we found no evidence for unfolded or degraded molecules after a long chase. Thus by these criteria, the molecule made in the presence of DTT can fold normally after removal of DTT.

In exploring the details of folding of HN protein synthesized in the presence of DTT, we did, however, observe two differences from the control. First, we found that folding of the molecule made in the presence of DTT was considerably slowed. This result suggests that cotranslational disulfide bond formation facilitates the folding of the molecule and argues that ordered formation of intramolecular disulfide bonds is important to the most expeditious folding of the molecule. Our results also showed that the folding pathway defined by conformationally sensitive monoclonal antibodies was altered for proteins made in the presence of DTT. While antigenic site 4 appeared first, similar to the control molecule, site 2, which appeared next to last and after sites 1, 3, and 14 in the control, formed prior to sites 1 and 3 in the DTT treated molecule. These results argue that the population of pulse-labeled molecules assumes a more mature conformation by a series of steps slightly different than the control. Formation of specific bonds on the nascent polypeptide chain may serve to anchor or lock in a specific localized conformation which facilitates subsequent folding of the molecule. Failure to form these bonds initially could lead to abnormally folded forms which would require rearrangement prior to initiation of a folding sequence. This scenario could account for slower appearance of antigenic sites. Also, disulfide bonds formed cotranslationally may lock in a particular conformation which would promote a specific folding sequence. Failure to set the pathway in one direction may result in an altered folding sequence.

Furthermore, the altered folding of the HN protein synthesized in the presence of DTT raised the possibility that this molecule was not folded normally after removal of DTT. Besides reactivity to conformationally sensitive antibodies, a sensitive determinant of a protein conformation is the activity of the protein. The neuraminidase and cell attachment activities of the mature protein found on cell surfaces was relatively insensitive to the effects of low concentrations of DTT as demonstrated in Fig. 8B. Indeed, it has been found that several proteins acquire DTT resistance after some folding has taken place (Braakman *et al.,* 1992). Thus to examine the effect of cotranslational presence of DTT on the activity of the

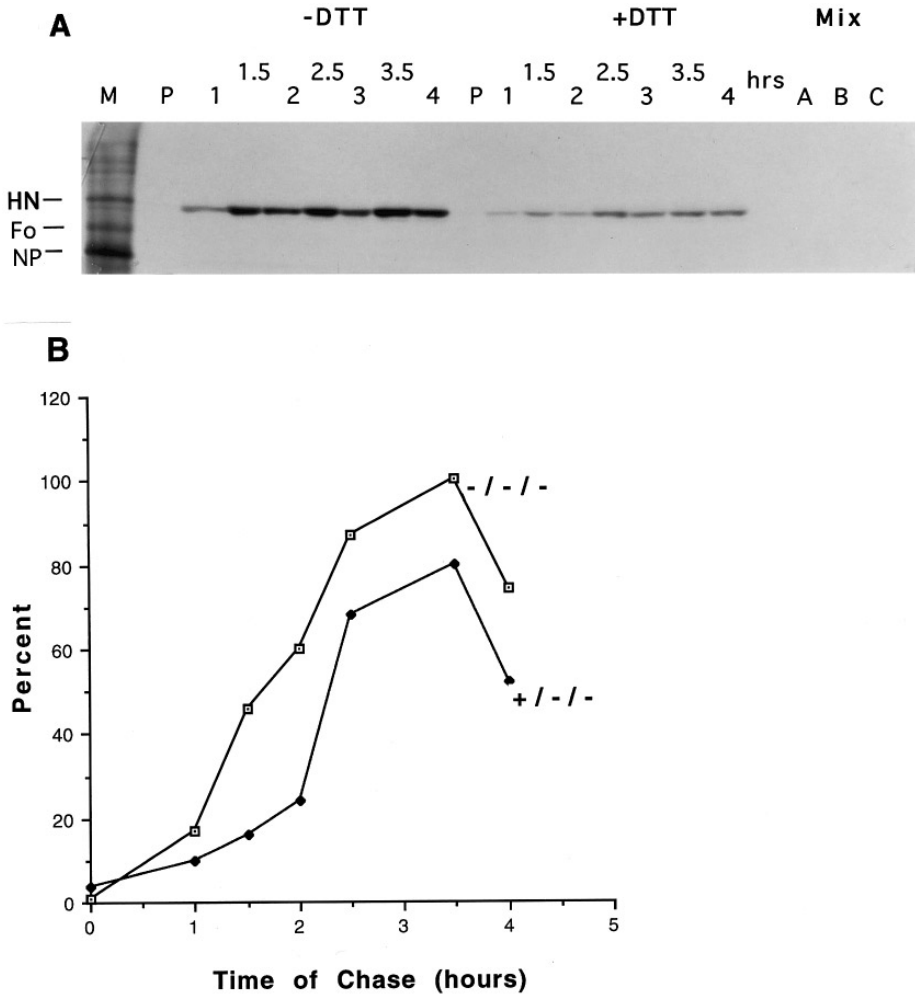

FIG. 9. Surface expression of HN protein. Infected cell monolayers were radioactively labeled in the absence or presence of DTT for 5 min and then subjected to various lengths of a nonradioactive chase in the absence of DTT as described in legend to Fig. 4. At various times of chase, intact cells were incubated with antibody and cell surface immune complexes isolated as described under Materials and Methods. (A) Results of one experiment. Each lane contains protein precipitated from 5×10^5 cells. Lane P, pulse label; M, marker proteins; 1–4, hours of chase; lane A, extracts from cells incubated with antibody but not labeled; lane B, extracts radioactively labeled but not incubated with antibody; lane C, a mixture of extracts from lanes A and B. (B) A quantitation of these results using the average of three separate experiments. The values for the +/−/− condition were normalized to account for a fourfold reduction in incorporation.

subsequently matured HN protein, DTT treatment was begun early in infection, at 4 hr, when only small amounts of protein have been made, and continued for 1.5 hr when viral protein synthesis increases significantly (Morrison and McGinnes, 1989). A chase of 2.5 hr allowed for folding and transit of molecules to the cell surface. While the positive controls, untreated infected cells as well as monensin-treated infected cells, showed significant neuraminidase activity and red blood cell binding, DTT-treated cells showed no evidence of either activity. Indeed, the activity associated with these cells was less than in cycloheximide-treated control cells. This result suggests that molecules synthesized in the presence of DTT were inactive and, therefore, conformationally abnormal. Indeed, in another system, there is a report that folding after DTT release results in a transit competent but biologically inactive protein (Simonen *et al.,* 1994).

Interestingly, we also found that molecules reduced with DTT just after synthesis were also affected in folding. The appearance of antigenic sites was slowed and their order of appearance was also altered. Disruption of bonds formed cotranslationally before final folding may result in a disordered structure similar to that found when bonds are inhibited cotranslationally.

Thus results suggest that cotranslational disulfide bond formation is important to subsequent folding of a conformationally normal NDV HN protein. The difference in conformation induced by forced posttranslational disulfide bond formation is unclear. Based on analysis of the location of changes in mutants selected to be resistant to monoclonal antibodies used here (Doms *et al.,* 1993; Iorio and Glickman, 1992; Iorio *et al.,* 1989a, 1989b, 1992, 1991; Morrison and Portner, 1991), the region of the molecule involved in antigenic site 2 is near the carboxy

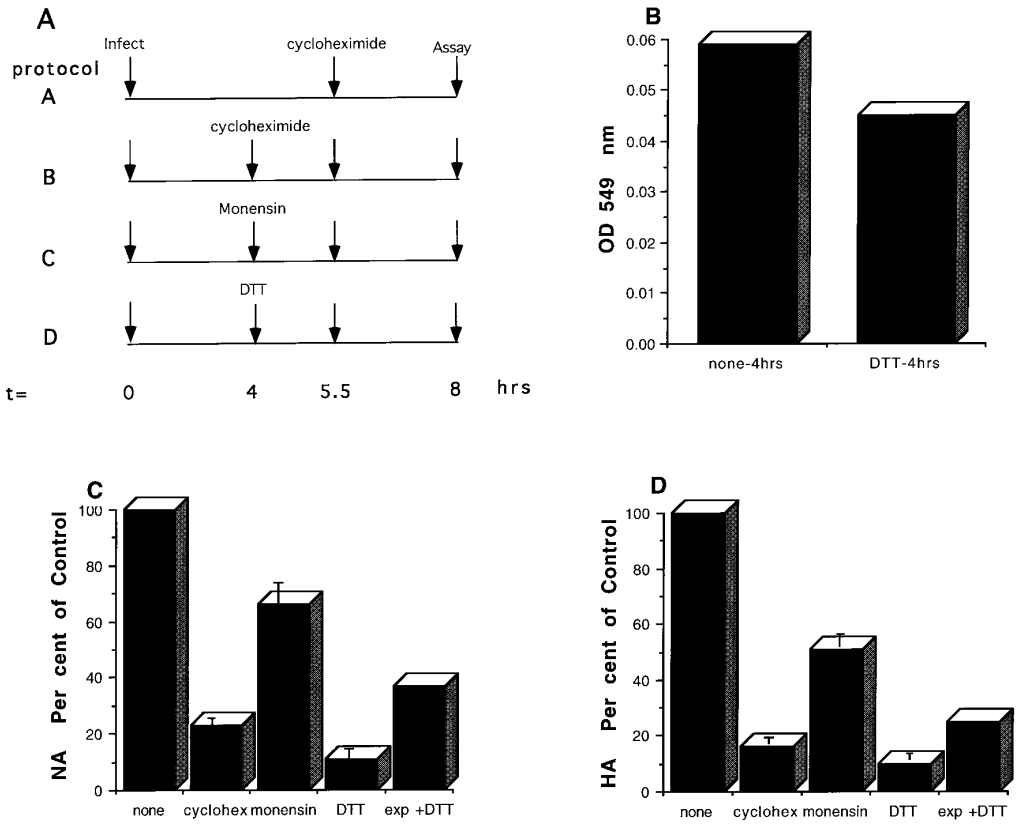

FIG. 10. Activity of the HN protein synthesized in the presence of DTT. Four monolayers of Cos cells (A–D) were infected with NDV and subjected to the different protocols outlined in A. In protocol A, cells were treated with cycloheximide (100 μg/ml) from 5.5 to 8 hr postinfection. In protocol B, cells were treated with cycloheximide from 4 to 8 hr postinfection. In protocol C, cells were treated with monensin (5×10^{-6} *M*) from 4 to 5.5 hr postinfection. Monensin was removed and cycloheximide was added for the remainder of the infection. In protocol D, cells were treated with DTT from 4 to 5.5 hr postinfection. After removal of DTT, cells were incubated in cycloheximide from 5.5 to 8 hours postinfection. (B) Neuraminidase activity at 4 hr postinfection without and with DTT treatment for 5 min. At 8 hr postinfection, the cell surface neuraminidase (C) and cell attachment (D) activities were determined as described under Materials and Methods. Results in C and D are presented as the percentage of untreated (protocol A) cells. The results are the average of four separate experiments. The actual variation is shown by error bars. The column labeled exp + DTT is that activity that would be expected from protocol D if all protein precipitable with conformationally sensitive antibody were biologically active.

terminal end of the molecule while site 4 is near the middle. Site 3 is located somewhat more amino terminal to site 4. Thus the carboxy terminus of molecules synthesized in the presence of DTT may fold earlier than in the normal situation. Under these circumstances it is possible that a prematurely folded carboxy terminus inhibits late tertiary changes required for a fully active molecule.

TABLE 1

Release of Infectious Virus after DTT Treatment

Protocol (treatment)	Plaque forming units/ml
Experiment 1	
A (None)	7.5×10^5
B (Cycloheximide)	5.1×10^4
C (Monensin)	1.6×10^5
D (DTT)	3.5×10^4
Experiment 2	
A (None)	2.6×10^5
B (Cycloheximide)	1.7×10^4
C (Monensin)	3.2×10^4
D (DTT)	1.2×10^4

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